

**REMARKS**

Claims 12-3, 17-20, 23-30, 33-35, 37, 39-43, 45-51, 53, 55-58, 61, and 65-66 are pending in this application. Claim 25 has been amended to clarify that the melting curve is generated by monitoring the fluorescence of the dsDNA binding dye. Support for this amendment is found throughout the specification. Claim 30 has been amended to remove YO-PRO®-1 and thiazole orange from the Markush group. Claims 29, 40 and 43 have been amended to change dependency. Minor amendments have been made to Claims 37 and 45. No new matter has been added by way of these amendments.

Claims 2-3, 17-20, 23-28, 30, 33-35, 37, 39-43, and 45-48 stand rejected under 35 U.S.C. § 102(b) as being anticipated by Wittwer et al. (U.S. Patent No. 6,174,670). According to the Examiner, Wittwer teaches a PCR reaction mixture comprising a target nucleic acid, PCR reagents, oligonucleotide primers configured for amplifying the target nucleic acid, and a dsDNA binding dye having a percent saturation of at least 50%. For disclosure of the dsDNA binding dye, the Examiner cites to col. 30, lines 61-67; col. 31, lines 1-4, and Fig. 15.

Claims 2-3, 17-20, 23, 25-28, 30, 33-35, 37, 39-43 all depend from independent claim 24, and claim 24 requires that the dsDNA binding dye has a percent saturation of at least 50%. “Percent saturation” is defined at page 4, lines 8-15 of the present application. For a given dye provided at maximum PCR compatible concentrations, the percent saturation is “the percent fluorescence compared to fluorescence of the same dye at saturating concentrations, i.e., the concentration that provides the highest fluorescence intensity possible in the presence of a predetermined amount of dsDNA.” Thus, the percent saturation is a ratio (expressed as a percentage) between the fluorescence at maximal PCR compatible concentration and the fluorescence at the concentration at which the dye reaches its maximal fluorescence intensity. Wittwer at col. 30, lines 61-67; col. 31, lines 1-4, and Fig. 15 discuss the use of the dsDNA binding dye SYBR Green I. As shown in Table 1, page 45 of the present disclosure, SYBR Green I has a percent saturation of 46%. Further, as discussed in the paragraph spanning pages 1 and 2 of the present application, it is known that SYBR Green I inhibits PCR at saturating conditions. Applicants respectfully submit that SYBR Green I does not have a percent saturation of at least 50%.

The Examiner cites to Fig. 15 and its description at col. 15, lines 1-5 of Wittwer for a teaching of a binding dye saturation of at least 80% or at least 100%. While Fig. 15 of Wittwer shows 100% fluorescence for each of the curves, applicants respectfully submit that this fluorescence level has nothing to do with percent saturation of the particular dye used. As discussed at col. 30, lines 66-67 of Wittwer, Fig. 15 shows normalized data; that is data normalized to the percent maximal fluorescence of each amplification. The formula for this normalization is even shown in Fig. 15 itself, where each data point is shown as the difference between the measured fluorescence ( $F_{\text{measured}}$ ) and the background ( $F_{\text{min}}$ ), divided by the difference between the maximal fluorescence for that sample ( $F_{\text{max}}$ ) and the background ( $F_{\text{min}}$ ), with the result multiplied by 100. Any curve so normalized will necessarily have a zero value and a 100 value, regardless of how much dye is used and the dye's percent saturation. In other words, Fig. 15 shows the percent relative fluorescence of 11 particular amplification curves, each curve normalized to its own maximum and minimum fluorescence. Fig. 15 does not give any information regarding the percent saturation of the dye used to generate the curve.

Furthermore, the amount of SYBR Green I dye used to generate the data in Fig. 15 was far from 80% or 100% saturating. As shown in Table 1, page 19 of the present specification, the maximal PCR compatible concentration of SYBR Green I is 1:5000, which equates to a percent saturation of 46%. The amount of SYBR Green I dye used in each amplification shown in Fig. 15 is a 1:10,000 dilution (see col. 30, lines 36-38, which references the reagents and conditions described in Example 2 (col. 22, lines 54-55)), only half the maximal PCR compatible concentration and only about 23% of the SYBR Green I dye needed to reach saturating concentrations. Thus, applicants respectfully contend that the data represented in Fig. 15 were not generated using SYBR Green I at saturating conditions, and that Fig. 15 shows normalized fluorescence, not percent saturation of the dye. Accordingly, applicants submit that the use of SYBR Green I in PCR analysis cannot be said to anticipate claim 24 or its dependent claims 2-3, 17-20, 23, 25-28, 30, 33-35, 37, 39-43.

With respect to claims 18-19, the Examiner cites to Wittwer, col. 22, lines 54-60. It is noted that claims 18 and 19 require two different sets of excitation and emission ranges. One set specifies the ranges of the fluorimeter, and the other set specifies ranges for maxima of the dye. While the cited section of Wittwer teaches similar excitation and emission ranges for SYBR Green I as required by one of the sets of ranges in claim 18, these similar ranges in

claim 18 are for the fluorimeter, not for the dye. Claim 18 requires the dye to have an excitation maximum in the range of 410-465 nm and an emission maximum in the range of 450-500 nm. These dye excitation/emission ranges are very different than the three dyes discussed at col. 22, lines 54-60. As seen in Table 1 on page 45 of the present disclosure, SYBR Green I has a excitation maximum of 489 nm and an emission maximum of 510 nm, both outside the dye ranges required by claim 18. Ethidium bromide similarly has excitation and emission spectra outside the range (518 and 605, respectively). While not shown in Table 1 of the present application, when bound to DNA, acridine orange has an excitation maximum of around 502 and an emission maximum around 525. Thus, it cannot be said that the disclosure of Wittwer, col. 22, lines 54-60 anticipates claims 18-19. Further, as discussed on page 4, line 25 through page 5, line 8 and Example 9 of the present application, it is not the individual excitation and emission ranges of the instrument or of the dye that is claimed, but rather the combination of monitoring using the green channel found on many PCR instruments, but using a blue dye, for which the excitation and emission maxima seem poorly matched. It was the finding that such blue dyes can produce sufficient signal, even in the green channel, that is surprising and inventive. It is further noted that claims 18 and 19 depend from claim 24, and the arguments made above with respect to claim 24 apply to these claims as well. Applicants respectfully submit that Wittwer does not anticipate claims 18-19.

With regard to claim 23, the Examiner cites Wittwer col. 14, lines 30-67; col. 15, lines 1-9; and col. 69, lines 11-55 for disclosure of mutation scanning on first and second samples and comparing melting curves. However, the disclosure cited by the Examiner uses a probe and measures fluorescence emission from the probe. Claim 23 requires detecting emission from a dsDNA binding dye. It is further noted that claim 23 depends from claim 24, and the arguments made above with respect to claim 24 apply to this claim as well. Applicants respectfully submit that Wittwer does not anticipate claim 23.

With regard to claims 25-28 and 37, the Examiner cites Wittwer col. 71, lines 15-67; col. 17, lines 1-46; and Figs. 13-26 for teaching methods comprising normalized magnitude of melting curve and repeating the normalizing steps with at least one additional target nucleic acid and plotting the difference between normalized curves.

Respectfully, claims 25-28 and 37 all require normalizing the magnitude of the melting curve. Melting curve normalization is described in Example 10 at page 39, lines 8-23 of the present disclosure. Melting curve normalization corrects for magnitude differences

between individual samples. Such normalization can be seen by comparing Figs. 12A and 12B of the present disclosure.

Respectfully, claim 59 at col. 71, lines 15-67, discloses derivative peaks of melting curves. Derivative peaks are the first derivative (or negative first derivative) of the melting curve. Such an example of derivative peaks are shown in Fig. 41B of Wittwer, as compared to the melting curves shown in Fig. 41A. Clearly derivative peaks are different than normalization of melting curves. With respect to Figs. 13-26 of Wittwer, as described at col. 17, lines 1-43, these all relate to amplification curves, not melting curves. Accordingly, the cited portions of the Wittwer disclosure do not teach normalization of melting curves.

Finally, as claims 25-28 and 37 depend from claim 24, the arguments presented above with respect to claim 24 apply to these claims as well. Applicants respectfully submit that Wittwer does not anticipate claims 25-28 and 37.

With respect to claim 30, the Examiner cites Wittwer, col. 11, lines 26-38 and col. 71, lines 8-12 for disclosure of SYBR Green, YO-PRO-1, and acridine orange. However, claim 30 does not include SYBR Green and acridine orange in the Markush group, and claim 30 has been amended to remove YO-PRO-1 from the Markush group. It is further noted that claim 30 depends from claim 24, and the arguments presented above with respect to claim 24 apply to these claims as well. Applicants respectfully submit that Wittwer does not anticipate claim 30.

With respect to claims 33-34, the Examiner cites Wittwer, col. 68, lines 10-67 for the disclosure of FRET labeled probes and the step of monitoring fluorescence from FRET probes where the target is no greater than 25 nucleotides.

Claims 33-34 require the dsDNA binding dye of claim 24, from which they depend, in addition to one fluorescently labeled probe. Respectfully, the disclosure of at Wittwer, col. 68, lines 10-67, claims using two probes wherein each probe is labeled with one member of a fluorescence resonance energy pair. There is no disclosure in Wittwer, col. 68, lines 10-67 of using a dsDNA binding dye or in using a fluorescently labeled probe that accepts fluorescence energy transfer from a dsDNA binding dye. Further, as claims 33-34 depend from claim 24, the arguments presented above with respect to claim 24 apply to these claims as well. Applicants respectfully submit that Wittwer does not anticipate claims 33-34.

With respect to claim 39, the Examiner cites Wittwer, col. 30, lines 30-67 and col. 31, lines 1-4, for disclosure that the amplifying and monitoring occur in a closed tube and no

reagents are added to the tube subsequent to initiation of amplification. As claim 39 depends from claim 24, the arguments presented above with respect to claim 24 apply to claim 39.

Applicants respectfully submit that Wittwer does not anticipate claim 39.

With respect to claims 40-43, the Examiner cites Wittwer, col. 40, lines 1-65; col. 43, lines 22-67; and col. 44, lines 1-18 for a teaching that the monitoring step comprises melting curve analysis occurs during and post amplification. Respectfully, the disclosure of Wittwer at col. 43, lines 22-67 and col. 44, lines 1-18 relates to an example in which probes are used for melting curve analysis. The use of probes for melting curve analysis is very different from the use of the dsDNA binding dye required by claims 40-43. Probes have specificity for a particular region of the amplicon, and the melting of the probe is indicative of the specificity of the probe for that region of the amplicon. On the other hand, dsDNA dyes bind with any double-stranded nucleic acid and, if not used at saturating conditions, can redistribute from one melting domain to another during melting of the amplicon. With respect to the disclosure at col. 40, lines 1-65, a dsDNA binding dye, SYBR Green I, is used. However, as discussed above, SYBR Green I does not have a percent saturation of at least 50%, as required by claim 24 and dependent claims 40-43. Further, as discussed at col. 40, lines 48-55 of the Wittwer reference, the post-PCR melting was accomplished by mixing purified PCR products and then melting. Claims 41-42 require melting during PCR, and claims 40 and 43 have been amended to require amplification and monitoring in a closed tube, without the addition of any reagents subsequent to initiation of amplification.

Applicants respectfully submit that Wittwer does not anticipate claims 40-43.

With respect to claim 43, the Examiner cites Wittwer, col. 67, lines 13-60 for the teaching that the monitoring step comprises determining whether the target has the same sequence as a second nucleic acid. However, Wittwer, col. 67, lines 13-60 is a claim directed to the use of a probe for melting curve analysis. As discussed above, the use of probes for melting curve analysis is very different from the use of the dsDNA binding dye required by claim 43. Accordingly, applicants respectfully request withdrawal of this rejection.

With respect to claims 45-48, the Examiner cites Wittwer, col. 71, lines 15-67; col. 17, lines 1-46; and Figs. 13-26 because according to the Examiner, Wittwer teaches the method of normalizing a melting curve, repeating the mixing and amplifying steps, comparing the normalized melting curves, and plotting the difference between the curves.

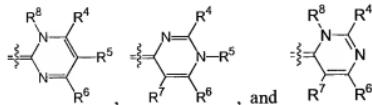
Respectfully, col. 71, lines 15-67 of Wittwer is a claim directed to the use of a pair of fluorescently labeled probes in melting curve analysis. As discussed above, the use of probes in melting analysis is very different from the use of dsDNA binding dyes, as required by claims 45-48. Additionally, the claim at col. 71, lines 15-67 requires derivative peaks of melting curves. As discussed above, derivative peaks are the first derivative (or negative first derivative) of the melting curve, which is not the same as a normalized melting curve. With respect to col., 17, lines 1-46 and Figs. 13-26, applicants note that Figs. 14-19C, 20-23 and 25 all show amplification curves or portions thereof, and Figs. 19D, 24, and 26 show data generated from amplification curves. Fig. 13 shows a comparison of an exo- and an exo+ polymerase with FRET hybridization probe pairs. Thus, while most of these figures relate to amplification curves, none of them shows melting curves, normalized or otherwise. Accordingly, Wittwer does not anticipate claims 45-48 and applicants respectfully request withdrawal of this rejection.

Claims 29, 49-51, 53, 55-58, 61, and 65-66 stand rejected under 35 U.S.C. § 103(a) as obvious over Wittwer in view of Haugland et al. (WO 00/66664). According to the Examiner, Wittwer teaches the methods of PCR analysis described in the rejections discussed above, but does not teach the dsDNA binding cyanine dyes as claimed in claims 29, 49-51, 53, 55-58, 61, and 65-66. According to the Examiner, Haugland teaches dsDNA binding dyes as cyanine dyes for their use in PCR reactions. Thus, the Examiner concludes that it would have been *prima facie* obvious to modify the method of Wittwer by using a cyanine binding dye as taught by Haugland, for the purpose of enhancing sensitivity of the detection of a target nucleic acid.

Claim 29 has been amended to depend from claim 40. As discussed above with respect to claim 40, Wittwer does not teach post-PCR melting in a closed tube format using a dsDNA binding dye having a percent saturation of at least 50%. Accordingly, Wittwer does not anticipate claim 29.

With respect to claims 49-51, 53, 55-58, 61, and 65-66, while Haugland teaches various cyanine dyes, Haugland teaches these dyes for staining electrophoretic gels, as well as detecting dsDNA in solution, Haugland does not teach that the dyes may be used in PCR reactions. Many dsDNA dyes interfere with the PCR reaction or fail to stand up to PCR conditions. Applicants were unable to find any suggestion in Haugland that the disclosed dyes are suitable for use in PCR. Furthermore, the dyes of Haugland are not the same

cyanine dyes as required in claims 49-51, 53, 55-58, 61, and 65-66. In claims 49-51, 53, 55-58, 61, and 65-66, the Q moiety must be one of the following heterocycles:



Each of these claimed pyrimidium heterocycles have two nitrogen atoms in the ring structure. In Haugland, the Q moiety may be a pyridinium (one ring) or a quinolinium (two rings). However, the Q moiety for all of the cyanine dyes disclosed on Haugland have only one nitrogen in the Q ring structure. Thus, not only does Haugland fail to disclose the suitability of its dyes for PCR, neither Haugland nor Wittwer teaches or suggests the specific compounds as required by claims 49-51, 53, 55-58, 61, and 65-66. Applicants respectfully request withdrawal of this rejection.

Applicants believe this response is timely made, and does not require the payment of any fee. However, in the event Applicants have inadvertently overlooked the need to petition for an extension of time or to pay an additional fee, Applicants conditionally petition therefor, and authorize any fee deficiency to be charged to deposit account 09-0007. When doing so, please reference the above-listed docket number.

If the Examiner has any questions, please contact the undersigned.

Respectfully submitted,

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